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ANALYSIS OF ALPHA INTEGRINS FOR THE DIAGNOSIS OF DIABETIC NEPHROPATHY

Background of the Invention

Diabetic nephropathy is a major cause of renal failure in the U.S. and develops in approximately 30% of insulin dependent diabetes mellitus (IDDM) patients. Recent studies by the Diabetes Control and Complications Trial Group have indicated that intensive insulin treatment substantially reduces the risk of developing complications, including nephropathy. However, the cost and effort of the intensive therapy, as well as the danger of hypoglycemic attacks dictate that this treatment should be limited to those patients who are prone to develop complications. It follows that an early selection of these diabetic subjects would be extremely helpful, but currently there are no adequate predictors available for clinical use.

Metabolic imbalance caused by hyperglycemia has been implicated as a major factor in the development of this condition and is associated with a genetic tendency to develop nephropathy. A prominent expansion of the mesangium with changes in the composition of the mesangial matrix have been observed in diabetic nephropathy (Williamson et al., *Diabetes Met. Rev.* 4:339 (1988), Steffes, M.W., et al. *Diabetes* 38:1077-81 (1989)).

Studies performed with human and experimental animal mesangial cells cultured in high-glucose medium have demonstrated an increased synthesis and accumulation of matrix proteins, namely collagens, including collagen type IV and fibronectin. This suggests that hyperglycemia plays a role in the mesangial changes of diabetic nephropathy. Ayo, S.H., et al. (1990a), *Am. J. Pathol.* 136:1339-1348; Nahman, N.S., et al., *Kidney Int.* 41:396-402 (1992); Danne, T., et al., *Diabetes* 42:170-177 (1993). The changes in the matrix secretion pattern of the cell are mediated either directly by hyperglycemia or by the glycation of mesangial matrix on prolonged exposure to high levels of glucose. Studies have demonstrated that cultured mesangial cells are influenced by the glycation of matrix leading to altered cell adhesion, spreading and proliferation. Since collagen IV (cIV) is the major component of the mesangial matrix (about 60%), changes in the interactions between this major mesangial glycoprotein and mesangial cells may play an important role in the pathology of diabetic nephropathy. Kim, Y., et al., *Am. J. Pathol.* 138:413-420 (1991). The changes in matrix deposition

are secondary in time to insulin insufficiency. Altered matrix deposition including basement membrane thickening is also found in a variety of arterioles and arteries in patients with diabetes mellitus. Altered matrix deposition is found in the pancreas of diabetic patients. Altered matrix deposition puts diabetic patients at risk for developing secondary pathological changes including, but not limited to nephropathy, myocardial infarction, cerebral stroke, problems associated with reduced circulation, retinopathy, neuropathies and the like.

Cell-matrix interactions are mediated, for the most part, by a family of receptors known as integrins. The very late antigen (VLA) subgroup of integrins which share a common $\beta1$ chain, include the cell membrane receptors for cIV, $\alpha1\beta1$ and $\alpha2\beta1$. Although integrins are mainly studied for their role in cell differentiation, migration and signaling events, they may also be involved in the maintenance of tissue structure. For instance, cells can modify their matrix by altering the production of matrix proteins and/or by regulating matrix organization. Cells cultured under high glucose conditions resulted in an increased production of matrix components by mesangial cells. (Kashgarian, M., et al., *Kidney Int.* 41:524-529 (1992).) The balance of cell surface integrin expression has been demonstrated to be altered in various disease states including inflammation and malignancy (Waes and Carey, *Otolarnyngologic Clinics of North America* 25(5):1117 (1992); Adams, J.C., et al., *Cell* 63:425-435 (1990); Rozzo et al., *FEBS Letters* 332:263 (1993)). This altered expression has been associated with altered adhesion to extracellular components.

Presently, the only earliest available indicator of kidney changes is microalbuminuria which occurs after the appearance of nephropathic changes. Yet only a percentage of individuals with microalbuminuria go on to develop glomerulopathy. Individuals at risk for developing glomerulopathy are best treated with intense glucose-modulating therapies that have their own risk. Often physicians are hesitant to place individuals with microalbuminuria on such therapies since the majority of these patients do not proceed to glomerulopathy. Biopsies indicating the accumulation of matrix accompanying the expansion of the mesangium occur at a point when the process has become irreversible. Therefore an early predictor of nephropathy or other disease states associated with altered matrix deposition would be beneficial as an indicator of those

patients who require stringent control of blood glucose levels to minimize nephropathic and other altered matrix deposition-associated disorders.

Thus, there is a need to identify markers associated with the changes seen in nephropathy and in other altered matrix deposition-associated disorders for the diagnosis of these disorders. There is a need to identify changes in regulation and function of integrins in diabetic patients and there is a need to develop a diagnostic test that can be used to identify patients who are likely to develop or have the early symptoms of nephropathy.

Summary of the Invention

Alterations in the amounts and patterns of alpha-integrin subunits has now been correlated to the onset of nephropathy. Analysis of alpha integrin subunit expression as compared with controls provides a diagnostic tool for the determination of patients likely to develop severe nephropathy and a method to monitor progress of disease during treatment protocols.

Cells that express alpha integrins, such as kidney tissue, fibroblasts, endothelial cells, and blood cells are analyzed for alpha integrin subunit expression, for example, by *in situ* hybridization methods. Changes in the amounts and pattern of integrin subunit expression as compared with control samples, is diagnostic of nephropathy and can be used to screen individuals, e.g., diabetic patients at risk for developing severe disease.

Analysis of $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$, and beta-1 integrin subunit expression as compared with control tissue expression is preferred. An increase in $\alpha 2$, $\alpha 3$, $\alpha 5$, or beta-1 integrin expression and/or a decrease in $\alpha 1$ expression is diagnostic of increased risk of nephropathy. An especially preferred diagnostic method is the comparison of $\alpha 1$ and $\alpha 2$ integrin subunit expression with control tissue. A pattern change including a decrease in $\alpha 1$ and an increase in $\alpha 2$ is diagnostic of increased risk of nephropathy or onset of the disease.

Brief Description of the Drawings

Figure 1 is a histogram summarizing results of *In situ* hybridization studies of rat control and diabetic tissue with $\alpha 1$ and $\alpha 2$ integrin probes.

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Detailed Description of the Invention

Analysis of changes in the pattern of integrin subunit expression, particularly of alpha integrin subunits, is made by comparing expression in sample tissues as compared with tissue controls.

Tissue Samples:

The invention is directed to methods of detecting changes in α integrin subunit expression in cells, such as the cell populations (visceral epithelial, endothelial and mesangial and other matrix-producing cells) present in the glomerulus; and also in the tubules as well as including, but not limited to, fibroblasts (for example see D. Kyu Jin, et al. in *J. Am. Society of Nephrology*, 5(3): 966, 1994), epithelial, and endothelial cells from a variety of tissues and organs as well as blood cells including, but not limited to polymorphonuclear leukocytes, monocytes, and the like. Changes to blood cells, including leukocytes, have been reported in diabetic patients who develop nephropathy (Ng, et al. *Diabetologia* 33:278-284, 1990).

A change in the expression of $\alpha 1$ and $\alpha 2$ integrins has been detected in the studies disclosed here, under conditions of high glucose (i.e., about 25 mM) compared with low glucose (i.e., about 5 mM), in diabetic test animals *in vitro*, and in a human diabetic patient with neuropathy. Mesangial cells cultured in high glucose showed an increase in $\alpha 2$ integrin expression and a decrease in $\alpha 1$ integrin expression compared with mesangial cells grown under low glucose conditions. A change in expression of α integrins such as $\alpha 1$ and/or $\alpha 2$ subunits can be used to identify patients that have or will develop diabetic nephropathy. In view of these studies, it is believed that patients showing about a 25 to 100% decrease in $\alpha 1$ integrin and/or about a 25 to 100% increase in $\alpha 2$ integrin expression have a greater chance of developing diabetic nephropathy. The methods disclosed here are useful to identify diabetic patients at risk for developing diabetic nephropathy. The methods may also be useful to monitor progression of diabetic nephropathy. Patients identified as having a risk for developing or showing early symptoms of diabetic nephropathy can be placed on a strict glucose control regimen so that the development and/or progression of nephropathy can be inhibited.

Changes in integrin subunit expression in diabetic patients have been identified in cultured human skin fibroblasts taken from skin biopsies (D. Kyu Jin, et al., *J. Am.*

Soc. of Nephrology 5(3):966, 1994) suggesting that a variety of integrin-expressing cells could be monitored to identify individuals with a predisposition to nephropathy or to other complications associated with diabetes-induced altered matrix deposition.

Methods of Detecting a Change in Expression of α1 and/or α2 Integrin Subunits in Cells from Diabetic Patients

The methods of the invention are conducted with cell types that express alpha (α) integrin subunits. Preferably, to identify patients predisposed to nephropathy, the cells are obtained from tissue samples from biopsy of kidney tissue of diabetic patients. However, other cell types that express α integrin subunits can be utilized including, but not limited to, fibroblasts, endothelial cells, polymorphonuclear leukocytes, monocytes, and other blood cells. The amount of cells typically obtained is relatively small so that the detection methods selected are those that can detect and/or quantitate α integrin subunit expression in a small cell sample. These methods include, but are not limited to *in situ* hybridization, including polymerase chain reaction (PCR) enhanced *in situ* hybridization (also known as *in situ* PCR) and the like.

The cell samples are obtained from patients having diabetes but having no demonstrable symptoms or signs of nephropathy. The earliest change in nephropathy is the detection of microalbuminuria. Biopsy specimens may also be obtained from diabetic patients that may have early symptoms of nephropathy so that the progression of diabetic nephropathy can be monitored. Blood samples and skin biopsies also can be obtained from patients with diabetes and processed for either *in situ* hybridization or PCR enhanced *in situ* hybridization (also known as *in situ* PCR). Similarly, it is possible to perform *in situ* hybridization or PCR enhanced *in situ* hybridization using a cheek scraping or a scraping of other accessible tissue.

Biopsy tissue samples are usually about 1mm^3 and are obtained using standard biopsy methods. Where the kidney is the organ selected for biopsy, kidney tissue from the cortical region is preferred although biopsy samples can be obtained elsewhere. Fibroblasts can be obtained from skin or any other tissue. The biopsy samples are then frozen in liquid nitrogen or fixed in 4% fresh paraformaldehyde and sectioned into 5 μm thick sections on silane-coated slides. The sections can then be treated with reagents to detect and/or quantitate α integrin expression in cells.

Blood cells and other α integrin expressing cells can also be analyzed for changes in α integrin subunit expression. These cells include fibroblasts, monocytes, polymorphonuclear leukocytes and other blood cells. Cells can be obtained and isolated from a blood or bone marrow sample. Methods for isolating particular cell types from a blood sample are well known in the art. Preferably leukocytes are isolated from blood by centrifugation, followed by hypotonic shock of residual blood cells as disclosed by Ng, et al. *Diabetologia* 33:278-284, 1990.

Rather than preparing cell sections, the sample of cells can be extracted to obtain nucleic acids using standard methods. The nucleic acids encoding α1 and/or α2 integrin subunits can be amplified using any of a variety of polymerase chain reaction methods. For example, changes in the level of expression of α1 and/or α2 integrins can be detected using a competitive PCR method as described by Gilland, G., *Proc. Natl. Acad. Sci. (USA)* 87:2725 (1990).

In a method of the invention, the level of α1 integrin expression is detected and/or quantitated in cells such as glomerular and tubular kidney cells. The level of α1 integrin expression can be detected using a variety of standard methods. The preferred methods are *in situ* hybridization, *in situ* PCR for detection of integrin RNA and immunofluorescence detection of antibody-tagged integrin protein. A decrease of about 25 to 100% in α1 integrin expression can indicate that early changes of diabetic nephropathy are occurring and can be used to identify patients that have an increased risk of developing diabetic nephropathy. A decrease in α1 integrin expression is compared to the level of α1 integrin expression in cells from age matched non-diabetic controls.

For detection and quantitation using *in situ* hybridization, the following method is preferred: a detectably labeled probe that is complementary to and/or hybridizes to all or a portion of nucleic acid sequences encoding all or a portion of α1 integrin subunit is utilized. A radioactively labeled probe preferably has a specific activity of about 2×10^8 to 1×10^9 dpm/μg. *In situ* hybridization on cells such as kidney tissue can be conducted as follows. 5μm tissue sections, fibroblasts and/or blood cells on silane-coated slides are further fixed in fresh 4% paraformaldehyde for 10 min. The slides are then pretreated with 0.2N HCl for 20 min., 0.05 M Triethanolamine (TEA, Sigma) for 15 min, 0.005% digitonin for 5 min., 3 μg/ml proteinase K (Sigma) for 15 min. at 37°C,

and 0.3% acetic anhydride - 0.1M TEA for 10 min. Hybridization is performed at 50°C overnight in 50% formamide, 0.6 M NaCl, 1xDenhardt's, 0.17 μg/ml human COT[RT] DNA (GIBCO/BRL), 1 mg/ml poly A (Boehringer Mannheim), 10% (W/V) Dextran sulfate (Sigma), 0.1 M dithiothreitol (DTT, Boehringer Mannheim), 1 mM EDTA, 0.1 mM aurinitricarboxylic acid (ATA, Sigma) and S^{35}-dCTP labeled cDNA probe. The following day, the slides are washed in 2x SCC-0.05% SDS for 60 min. at 55°C; further washed in the high stringency washing buffer containing 50% formamide, 0.6 M NaCl, 1 mM EDTA, 5 mM DTT and 10 mM Hepes for 4 days at room temperature. After 4 days, the slides are rinsed in 2x SCC and the slides are dehydrated in graded ethanol with 0.3 M ammonium acetate, then dipped in Kodak NTB-2 emulsion and exposed for 5 days at 4°C. After development, the slides are stained with hematoxylin-eosin (Surgipath Canada, Inc., Winnipeg, Canada) and mounted. The silver grain number per cell are used to quantitate the result of *in situ* hybridization. About 10-20 glomeruli and a similar number of tubules are examined per patient.

A probe of the invention hybridizes to and is complementary to and/or all or a portion of a nucleic acid sequence encoding $\alpha 1$ integrin as long as the probe specifically detects $\alpha 1$ integrin expression. Probes can be designed using a known sequence such as the rat $\alpha 1$ integrin sequence as shown as Figure 2 in Takada and Hemnlev, *J. Cell Biol.* 109:397-407 (1983) or by the use of commercially available programs and are capable of binding to rodent or human $\alpha 1$ integrin but are not capable of binding to other proteins including other proteins having regions homologous to α integrins when tested under identical hybridization conditions. Examples of other proteins that have homologous regions to α integrins include those proteins identified using a gene bank search, such as GenBank, or the like, or in publications related to $\alpha 1$ and $\alpha 2$ subunits (for example, see Ignatius, et al. *J. Cell Biol.* 111:709-720, 1990 listing proteins with homologies to the $\alpha 1$-subunit).

The probe can be about 15 nucleotides long up to a full length probe of about 4kb. The probes are preferably 100% complementary to the nucleic acid encoding $\alpha 1$ integrin however some mismatches can be present depending on the length of the probe. About 1 to 3 mismatches in a probe of about 20 to 30 nucleotides long can be present as long as hybridization conditions are adjusted to account for mismatches. Hybridization conditions can be adjusted to take into account mismatches in accord with known

principles as described in Sambrook et al., A Guide to Molecular Cloning, Cold Spring Harbor NY (1989).

A specific example of a nucleic acid sequence encoding $\alpha 1$ integrin is the rat $\alpha 1$ integrin sequence shown as Figure 2 in Ignatius et al., *J. Cell. Biol.* 111:709-720, 1990, (SEQ ID NO:1) and the protein sequence encoded by $\alpha 1$ integrin is provided as SEQ ID NO:2. A DNA sequence encoding $\alpha 1$ integrin can be obtained from a rat pheochromocytoma cell line PC12 as described by Ignatius et al., *J. Cell. Biol.* 111:709 (1990). Briefly, a cDNA library can be prepared from rat pheochromocytoma PC12 in a lambda vector. The sequence can be identified and/or amplified using probes or primers designed from the known sequences using standard methods as described in Sambrook et al., (*supra*). Once the sequence is subcloned it can be confirmed by sequence analysis and/or by screening with antibodies specific for $\alpha 1$ integrin. Other DNA sequences encoding $\alpha 1$ integrins can be identified and isolated using probes and primers derived from the known sequences.

A preferred probe is a 3.9 kb fragment from the 5' end through the EcoR1 site near base 3900 including the sequence as shown in Figure 2 of Ignatius et al. *(supra)*. Smaller fragments that can form probes can readily be prepared with restriction enzymes or derived by automated or manual oligonucleotide synthesis techniques, by PCR, or by other methods also known in the art. The probes are preferably detectably labeled with a radioactive nucleotide using standard methods.

Other methods utilizing probes for detection of $\alpha 1$ integrin expression can also be utilized using standard methods such as Northern Blot Analysis and the like as described in Sambrook et al., cited *supra*.

Primers can also be designed based upon the sequence of rat $\alpha 1$ integrin sequence. This invention also contemplates using primers and nucleic acid sequences from the human $\alpha 1$ integrin sequence provided by Briesewitz, et al. (*J. Biol. Chem.* 268(4):2989-96, 1993). Primers can be designed using a known sequence using commercially available computer programs. Primers typically are complementary to and/or hybridize to a 5' region and/or a 3' region of the nucleic acid sequence encoding the protein of interest. The primers can be used to amplify all or a portion of DNA or cDNA encoding $\alpha 1$ integrin. Primers can be used to make probes and to detect expression levels of $\alpha 1$ integrin. Primers preferably have at least 15 nucleotides that are

100% complementary to the nucleotide sequence selected. The primers can also have additional sequences preferably at the ends of the primer that include restriction enzyme sites and the like that are not complementary to the nucleic acid sequence to be amplified. Primers are preferably about 15 to 50 nucleotides long and can be prepared by automated synthesis.

The primers can be used to detect the level of $\alpha 1$ expression in cells. RNA from cells is extracted and reverse transcribed using standard methods. Primers that are complementary to and can hybridize to a DNA sequence encoding $\alpha 1$ integrins are utilized to amplify the cDNA. A decrease in the level of PCR product can be determined in comparison to the amount of PCR product obtained from control cells.

One method of utilizing PCR to detect $\alpha 1$ integrin expression is *in situ* PCR. A method for PCR *in situ* hybridization is described in PCR *In Situ* Hybridization Protocols and Applications, J. Novo ed., "PCR *In Situ* Hybridization", pp. 157-183. Briefly, tissue sections, fibroblasts and/or blood cells (about 5 μm) are placed on silane-coated glass slides. After removing paraffin, the slides are treated with trypsinogen (2mg/ml) in 0.01N HCl for 10 minutes and then trypsinogen inactivated in 0.1M Tris HCl (pH 7.0) solution. The slides are washed sequentially in 90% and 100% ethanol, two times for 1 minute each and air dried. Aliquots of reaction mixture containing 0.15 units/ml *Taq* DNA polymerase and specific primer pairs for $\alpha 1$ integrin are added to the tissue section and then overlaid with siliconized glass coverslips. The slides are placed in the heat-sealable plastic bags and 4-5ml mineral oil is added. After removing air, the bag is heat-sealed and placed in the thermal-cycling oven for 40 cycles. After thermal-cycling, the slides are washed twice in chloroform for 2 minutes. The coverslips are removed and the slides are dipped briefly in fresh chloroform. After washing in PBS for 5 minutes, the slides are dehydrated and air-dried. The slides are dipped in NTB2 nuclear emulsion (Kodak) and exposed in the dark for 7 days. After development, the slides are *counterstain*ed with hematoxylin-eosin.

A change in the level of $\alpha 1$ integrin protein expression can also be detected by using immunofluorescence. (Unless otherwise specified as "protein expression", the term "expression" used herein generally refers to RNA expression.) Sections of tissue samples, fibroblasts and/or blood cells can be stained with antibodies specific for $\alpha 1$ integrin. It is preferable that antibodies are monoclonal antibodies and are antibodies

that do not substantially cross-react with other α integrin subunits. Antibodies to α1 integrin can be made by standard methods such as described in Wayner EA and WG Carter, 1987, *J. Cell Biol.* 121(5):1141-1152. Antibodies specific to α1 integrin include the SR84 and TS2/7 antibodies. Information related to these antibodies is provided in Examples 1 and 3. A decrease in the level of immunofluorescence can be observed and quantitated using standard methods. A decrease of about 25 to 100% of α1 integrin expression may be used to identify patients that have a greater risk of developing diabetic nephropathy. A decrease in α1 integrin expression is compared to the level of α1 integrin expression in age-matched nondiabetic controls.

The preferred method of the invention involves comparing the level of expression of α2 integrin to the level of expression of α1 integrin. Under high glucose conditions, a decrease in the level of α1 expression is seen as well as an increase in the level of α2 expression in mesangial cells. It is believed that patients at greater risk for nephropathy or other complications associated with diabetes will exhibit an increase in α2 expression and a decrease in α1 expression. A change of about 15 to 100%, and preferably of about 25 to 100%, of α2 integrin expression as well as a change of about 15 to 100%, and preferably of about 25 to 100%, of α1 integrin expression is believed to be indicative of patients with a greater risk of developing diabetic nephropathy.

Integrin expression is associated with a variety of cell types in a variety of locations throughout the body, therefore it is possible that altered levels of integrin expression will also be identified in diabetic associated retinopathy, atherosclerosis and select diabetic neuropathies.

The expression of integrin subunits, preferably of α1 and α2 integrin subunits, is detected and/or quantitated in tissue samples, fibroblasts and/or blood cells from diabetic patients. The preferred methods are those that allow detection of gene expression in a small amount of cells or tissue.

The expression of α2 integrin can be detected using *in situ* hybridization. The conditions for *in situ* hybridization are the same as those described previously. A probe specific for nucleic acid sequences encoding α2 integrin can be prepared using standard methods as described in Sambrook et al., cited *supra*. The probes are complementary to and/or hybridize to all or a portion of a nucleic acid sequence encoding α2 integrin. As described for α1 integrin, the probe to detect α2 integrin can hybridize to a portion of a

nucleic acid sequence as long as the probe specifically detects a sequence encoding α2 integrin. Nucleic acid sequences can be DNA, cDNA, or RNA. It is preferred that the probe hybridize to RNA or cDNA.

A specific example of nucleic acid sequence encoding α2 integrin is shown in Figure 2 of Takada and Hemler, *J. Cell Biol.* 109:397 (1989). (SEQ ID NO:3). DNA sequence encoding human α2 integrin can be isolated as described in this reference. The protein encoded by SEQ ID NO:3 is provided in this disclosure as SEQ ID NO:4. Nucleic acid sequences encoding α2 integrin can be obtained from human lung fibroblasts and/or human endothelial cells. Preferably DNA libraries from endothelial cells can be prepared and nucleic acids encoding α2 integrin identified and/or amplified using probes and primers derived from the sequence of α2 integrin, e.g., as shown in Figure 2 of Takada et al. (*supra*). If primers are selected, DNA sequences can be amplified using the polymerase chain reaction and then subcloned. Clones that are positive by hybridization to a probe specific for DNA sequences encoding α2 integrin (see Examples 1 and 3) or that express proteins that are positive by reacting with an antibody specific to α2 integrin such as P1H5 are selected. A DNA sequence encoding α2 integrin can be confirmed by DNA sequencing in comparison to the known α2 sequence, as shown in Figure 2 of Takada et al. (*supra*).

A probe of the invention hybridizes to and is complementary to and/or hybridizes to all or a portion of a nucleic acid sequence encoding α2 integrin as long as the probe specifically detects α2 integrin expression. Probes can be designed using a known sequence such as shown in Figure 2 of Takada et al. (*supra*) by the use of commercially available programs.

The probe can be about 15 nucleotides long up to a full length probe of about 5Kb. The probes are preferably 100% complementary to the nucleic acid encoding α2 integrin however some mismatches can be present depending on the length of the probe. About 1 to 3 mismatches in a probe of about 20 to 30 nucleotides long can be present as long as hybridization conditions are adjusted to account for mismatches. Hybridization conditions can be adjusted to take into account mismatches in accord with known principles are described in Sambrook et al., A Guide to Molecular Cloning, Cold Spring Harbor NY (1989).

A preferred probe is a 1.8 fragment kb from the 5' end through the EcoR1 site near base 1800 of the sequence shown in Figure 2 of Takada et al. (*supra*). Other probes can be derived from this fragment or from the full length sequence by use of restriction enzyme digestion. Probes can also be prepared by automated synthesis or by PCR. Probes are preferably detectably labeled with a radioactive nucleotide using standard methods.

Probes specific for $\alpha 2$ integrin expression can then be utilized in methods of detecting $\alpha 2$ integrin expression in various cell types. The preferred method is by use of *in situ* hybridization or PCR-*in situ* hybridization on kidney as well as other tissues. The method utilized for *in situ* hybridization has been described previously (Takada and Hemler, *supra*). The method for PCR *in situ* hybridization has been described for $\alpha 1$ integrin. Other methods utilizing probes for detection of $\alpha 2$ integrin expression can also be utilized using standard methods such as Northern Blot Analysis, and the like, as described in Sambrook et al. cited *supra*.

Primers can also be designed based upon the known DNA sequence encoding human $\alpha 2$ integrin. Primers can be designed from a known sequence such as shown in Figure 2 of Takada et al. (*supra*), using commercially available software. Primers typically are complementary to and/or hybridize to a 5' region and/or a 3' region. The primers can be used to amplify all or a portion of DNA or cDNA encoding $\alpha 2$ integrin. Primers can be used to make probes and to detect expression levels of $\alpha 2$ integrin. Primers preferably have at least 15 nucleotides that are 100% complementary to the nucleotide sequence selected. The primers can also have additional sequence preferably at the ends of the primer that include restriction enzyme recognition sites and the like. Primers are preferably about 15 to 50 nucleotides long and can be prepared by automated synthesis.

Primers can be used to detect the level of $\alpha 2$ integrin expression in cells. Nucleic acids, preferably RNA, from cells from diabetic patients are extracted and reverse transcribed using a standard method. Primers that are complementary to and can hybridize to a cDNA sequence encoding $\alpha 2$ integrin are utilized to amplify the cDNA. An increase in the level of PCR product can be determined in comparison to the amount of PCR product obtained from control cells.

A change in the level of $\alpha 2$ integrin protein expression can also be detected by using immunofluorescence. Sections from kidneys and/or other tissues, skin fibroblasts and/or blood cells can be incubated with antibodies specific to $\alpha 2$ integrin. It is preferable that the antibodies are monoclonal antibodies and are antibodies that do not crossreact with other α integrin subunits. Antibodies to $\alpha 2$ integrin can be made by standard methods such as described in Wayner EA and WG Carter, 1987, *J. Cell Biol.* 121(5):1141-1152. Antibodies specific for $\alpha 2$ integrin include P1H5. An increase in the level of immunofluorescence can be observed and quantitated using standard methods such as flow cytometry. An increase of about 25 to 100% of $\alpha 2$ integrin expression can be used to identify patients that have a greater risk of developing diabetic nephropathy. An increase in $\alpha 2$ integrin expression is compared to $\alpha 2$ integrin expression in nondiabetic control cells.

An increase in $\alpha 2$ integrin expression alone can also be used to identify a patient that may have a greater risk of developing diabetic nephropathy. An increase in $\alpha 2$ expression can be determined as described using the methods described above. An increase of about 25 to 100% in $\alpha 2$ integrin expression may indicate a patient who has an increased risk of developing diabetic nephropathy.

Although an increase of $\alpha 2$ integrin expression or a decrease of $\alpha 1$ integrin expression alone can be utilized to identify patients at greater risk for developing diabetic nephropathy, a preferred method is to detect changes in both $\alpha 1$ and $\alpha 2$ integrin expression. It is believed that an increase in $\alpha 2$ integrin expression and a decrease in $\alpha 1$ integrin expression identifies patients that are at greater risk of or are showing early symptoms of diabetic nephropathy.

In one step of the method, the level of $\alpha 2$ to $\alpha 1$ integrin is compared. The level of $\alpha 1$ integrin expression can be detected and/or quantitated using the methods described previously. The level of $\alpha 1$ and $\alpha 2$ integrin expression can be quantitated on two different cell samples such as two sections of the same tissue sample. About 10-20 glomeruli and tubules are examined. On one cell sample containing the same type of cells, $\alpha 2$ integrin expression can be quantitated and on a second cell sample with the same type of cells, $\alpha 1$ integrin expression can be quantitated. Alternatively, the level of $\alpha 1$ and/or $\alpha 2$ integrin expression can be determined using the same cell sample if the agent used to detect $\alpha 1$ expression is detectably labeled with a first detectable label and

the agent used to detect α2 expression is detectably labeled with a second detectable label. The first detectably labeled agent and the second detectably labeled agent are agents selected that can be detected and/or quantitated in the presence of one another.

In a preferred version, kidney tissue sections taken from diabetic patients are fixed in formalin and then treated with HCl and proteinase K. A first probe specific for α1 integrin is a 3.9 kb fragment from 5' end through EcoR1 site near base 3900 probe including a sequence as shown in Figure 2 of Ignatius et al. (*supra*). This probe is labeled with ^{32}P or 35S or other suitable labels known in the art including, but not limited to, fluorescent labels, biotinylated labels, or other radio labels and the like. The probe is incubated with the section as described previously. A second section taken from the same tissue sample is treated in the same manner but incubated with a probe specific for α2 integrin expression. In a preferred embodiment, a probe specific for α2 integrin expression is a 1.8 kb fragment from 5' end through EcoR1 site near base 1800 that includes a sequence as shown in Figure 2 of Takada et al. (*supra*). Both probes are labeled with ^{32}P or ^{35}S. The probe is incubated with the section overnight at 50°C and then for 4 days at room temperature. The sections are then developed for autoradiography. The number of grains per cell are counted for about 10-20 glomeruli and tubules. The total counts for α2 integrin expression vs. α1 integrin expression are compared. An increase of about 40% in α2 integrin and a 30-40% decrease of α1 integrin may indicate a patient is at greater risk for developing diabetic nephropathy.

In an alternative version, the level of expression of α2 integrin is compared with the α1 expression which can be determined using *in situ* PCR or competitive reverse transcriptase PCR. Primers specific for α1 and α2 integrin expression can be prepared as described previously. For competitive reverse transcriptase PCR, RNA extracted from different cell types obtained from diabetic patients will be reverse transcribed to generate cDNA. The cDNA will be mixed with the various concentrations of competitive template amplified by the PCR method. After degradation of competitive cDNA with restriction enzyme, amplified cDNA will be subjected to electrophoresis in 2% agarose gel, electrotransferred to a nylon membrane, UV cross-linked to the membrane and hybridized with a ^{32}P-labeled probe. Autoradiographs will be used to quantify the label bound to the cDNA using amount of label bound to samples containing target cDNA alone as compared to samples also containing competitor

cDNA to arrive at the target cDNA concentration. For *in situ* PCR, a method has been described previously. The change in α1 and α2 integrin expression can be quantitated by counting the number of grains per cell in control vs. diabetic cells.

Optionally, for each of the detection methods for α integrin subunits, the level of integrin subunit expression can be compared to expression of a control. The control is selected to be a protein expressed at the same levels in both normal and diabetic cells. The control protein is also selected to be one that is expressed at sufficient levels for easy detection and quantitation. The level of expression of α1 and α2 integrin expression can each be compared to that of the level of the control RNA expression in the cells. The level of RNA expression of α1 integrin or α2 integrin can be divided by the level of expression of the control RNA to normalize the values to the level of control expression in a particular cell sample. The level of expression of the control protein is detected and quantitated using the same method as α1 or α2 integrin expression. The preferred control protein is a cell surface HLA determinant.

Optionally, the levels of α3, α5, or beta-1 integrin subunit expression can be analyzed as described above. The level of α3, α5, or beta-1 integrin expression in cells such as kidney tissue can be detected and quantitated as described for α1 and α2 integrin expression including *in situ* hybridization, *in situ* PCR, immunofluorescence and the like. Other cell types can be analyzed as described above, including fibroblasts and blood cells. Antibodies specific for α3, α5, and beta-1 can be prepared as described by Wayner et al. cited *supra*.

A DNA sequence encoding α3 integrin has been described in Takada et al., *J. Cell Biol.* 115:257 (1991). A probe specific for cDNA sequence encoding α3 integrin subunit is a 1.4Kb Sal I fragment containing 5' untranslated and amino terminal coding sequences for α3 subunit of integrin. DNA sequences encoding α3, α5, and beta-1 integrin can be utilized to form primers and probes as described previously.

The level of α3, α5, or beta-1 integrin expression is increased about 15 to 100% compared with cells from age matched nondiabetic controls. It is believed that an increase in α3, α5, or beta-1 integrin subunit expression may also identify patients that have an increased risk of developing diabetic nephropathy or that have early signs of diabetic nephropathy.

This invention also relates to methods for detecting alterations in integrin subunit expression, particularly $\alpha 1$ and/or $\alpha 2$ integrin subunit expression by obtaining a cell sample from a patient, processing the sample to detect alterations in integrin subunit expression as compared to integrin expression in samples from age matched normal controls, detecting levels of integrin expression and determining if these levels are altered relative to controls.

This method is useful for predicting individuals at risk for developing pathologies associated with altered cell matrix deposition, including but not limited to renal nephropathy. In preferred embodiments of this invention, the tissues used to detect altered $\alpha 1$ and/or $\alpha 2$ integrin expression include kidney biopsies, skin biopsies and blood cells including polymorphonuclear cells, monocytes, and other cells expression integrin subunits. Biopsied tissue can be further separated into its cellular components or processed as tissue sections for *in situ* hybridization techniques, and/or for immunodiagnostic techniques including immunofluorescence and immunoperoxidase staining.

The cellular components of the biopsied tissue can be cultured for *in vitro* studies including Northern procedures, PCR techniques, immunofluorescent techniques and/or in situ hybridization techniques. Alternatively, cells can be separated and analyzed by flow cytometry, immunofluorescence, processed for PCR or for any of a variety of techniques discussed throughout this disclosure.

While blood cell components are preferably separated from the whole blood sample using methods well known in the art. Individual cells are separated, where necessary, using techniques such as those of Ng, et al. (*supra*), and Baron, et al. *Clin. Sci.* 37:205-219, 1990. Preferably the samples are tested using *in situ* hybridization methods. Where the amount of tissue available is fairly small, PCR-enhanced *in situ* hybridization can be used.

The present invention is also directed to a kit to detect alterations in integrin subunit expression, particularly $\alpha 1$ integrin and/or $\alpha 2$ integrin subunit expression in a patient sample. A variety of kits are contemplated to encompass a variety of methods. These kits optionally include reagents to process a tissue or cell sample for the technique employed by that particular kit. By example, a kit for PCR or PCR enhanced *in situ* hybridization can include reagents to process the cell sample or section and

isolate the RNA (for PCR). It will also contain suitable primers to amplify the target sequence and additional probes, if necessary, to detect the desired nucleic acid fragments as well as buffers and reagents for the polymerase chain reaction and the buffers and emulsions required to develop the silver granules, and the like, for *in situ* hybridization methods. Other kits can alternatively include reagents for immunofluorescence using antibodies to the integrin subunits and/or probes, primers and reagents for modifications of *in situ* or PCR *in situ* hybridization methods.

All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference.

It will be apparent to one of ordinary skill in the art that many changes and modifications can be made in the invention without departing from the spirit or scope of the appended claims.

Example 1
Effect of High Glucose on the Synthesis and Cell Surface Expression of Integrin Receptors by Cultured Mesangial Cells

Cell lines and culture conditions

Human mesangial cells (HMC) were isolated from 19-22 week old fetal kidney tissue or adult tissue as previously described (Striker and Striker, *J. Lab. Invest.* 53(2):122-131, 1985). Cells were cultured at 37°C in an environment of 95% air and 5% CO_2 and in media composed of MEM (Sigma, St. Louis, MO) containing 5 or 25 mM glucose, 20% FBS, 15mM Hepes, penicillin (100 U/ml), streptomycin (100mg/ml), and amphotericin (25mg/ml). Cells were cultured in the two different conditions for at least two passages before they were used for experiments. Cells were released from their tissue culture flasks for passaging or for use in experiments, by washing twice with 1 mM EDTA in HBSS and then treating with 0.05% trypsin and 1 mM EDTA in HBSS for 1 min. Cells between passage 4 and 9 were used in experiments.

The cells were grown in T-75 flasks until 75-80% confluent. For the adhesion and immunoprecipitation analyses, cells were metabolically labeled for 18 hours with 0.5 mCi of [^{35}S]-methionine per T-75 flask. [^{35}S]-methionine was obtained from Du Pont/NEN, Boston, MA.

Monoclonal antibodies (Mabs) to integrin receptors

Mabs to the integrin receptors α3 (P3D11), α5 (P3D10) and β1 (P5D2) can be produced as previously described (Wayner et al., *J. Cell. Biol* 121(5):1141 (1993)) and are available from EA Wayner, Fred Hutchinson Cancer Center, Seattle, WA. The antibodies were characterized by sequential immunoprecipitation with known Mabs directed against these integrin receptors (P1B5, P1D6, P4C10) available from EA Wayner. Other Mabs α2 (P1H5), α4 (P4G9) and β2 (P4H9) were previously described (Wayner et al., cited *supra* 1993) and are available from EA Wayner, Fred Hutchinson Cancer Center, Seattle, WA. TS2/7 was provided by Dr. Martin Hemler (Dana Farber Cancer Institute, Boston, MA).

SR84 supernatant was used as a function-blocking anti-α1 Mab in inhibition experiments. SR84 is available from Dr. D.O. Clegg (Univ. of California, Santa Barbara, CA). (α6) G0H3 was purchased from AMAC Inc., Westbrook, ME. In addition monoclonal antibodies to α1 and α2 integrin were obtained from Telios Pharmaceuticals (San Diego, CA). Hybridoma culture supernatant or ascites fluid were used for immunoprecipitation, flow cytometry and inhibition experiments. A Mab directed to a cell surface HLA determinant was used as a negative control (W6/32, HB95: American Type Culture Collection, Rockville, Maryland, USA). W6/32 bound to the surface of cultured mesangial cells but did not influence adhesion of cIV. SP2 myeloma culture supernatant was also used as a control.

Immunoprecipitation analysis of integrins from mesangial cell membranes

Mesangial cells metabolically labeled with [^{35}S]-methionine were detached from flasks by treatment with trypsin (Sigma) for 2 minutes, washed three times with phosphate-buffered saline (pH 7.4) and resuspended in PBS containing protease inhibitors (1 mM PMSF and 1 mM NEM). The radiolabeled cell membrane proteins were solubilized by adding lysis buffer (1% Triton X-100, 1 mM Calcium, 1 mM

PMSF, 1 mM NEM and PBS at pH 7.4) and incubating for 60 minutes at 4°C. Insoluble material was separated by centrifugation at 10,000 rpm for 30 minutes.

The supernatant was transferred and 10 µl was tested for radioactivity ($\geq 10^7$ cpm/per antibody being assayed was considered to be adequate for immunoprecipitation). The lysate was precleared once with fetuin-agarose which was removed by centrifugation at 10,000 rpm for 15 minutes. This was followed by three preclears with protein A agarose bound to rabbit anti-mouse IgG, the last preclear was done overnight.

For immunoprecipitation, the cell lysate (equal counts of lysate for cells in 5 and 25 mM glucose were used) was incubated with the monoclonal antibodies to be tested, pre-bound to rabbit anti-mouse protein A-agarose. Myeloma culture supernatant was used as a negative control. Anti-HLA antibody (W6/32) was used as a control for loading. After an overnight incubation at 4°C, the agarose beads were washed five times and bound material was eluted by boiling for 5 minutes in SDS.

The eluted material was analyzed by loading lysate from each permutation on a 7.5% non-reducing SDS-PAGE gel and labeled proteins were visualized by autoradiography. The fluorograms were scanned with a Macintosh Quadra 840 computer using the NIH Image 5.1 Program, and the optical density of the bands was red after subtracting the background. The O.D. was corrected using the lanes immunoprecipitated with W6/32. Immunoprecipitation assays were performed three times for each growth condition of mesangial cells.

Immunoprecipitates were obtained with anti-integrin monoclonal antibodies from detergent extracts of metabolically labeled human kidney mesangial cells grown in 5 (low) or 25 mM (high) glucose. Equal counts of membrane proteins were immunoprecipitated to compare the level of integrin receptors of mesangial cells under the two growth conditions of low or high glucose levels.

Cells grown in 25 mM glucose have a higher specific activity of labeling than cells in 5 mM glucose. To overcome this difference and permit a comparison of the band intensity on immunoprecipitation equal counts of cell lysate from the two populations were immunoprecipitated with the antibody. Densitometry and statistical analysis of three experiments was performed, the data normalized to the HLA control and expressed as an O.D. ratio of cells grown in high glucose (HG) to cells grown in

low glucose (LG), for three experiments, with (LG = 1). Cells were labeled with [^{35}S]-methionine, the cells were harvested, and solubilized. Samples were incubated with antibody and equal counts of cell lysate from the two cell populations were immunoprecipitated with equal amounts of antibody.

The control indicated that there were comparable amounts of cell surface HLA determinant precipitated from each sample. W6/32, a Mab to cell surface HLA determinant was used as a negative control. Other antibodies used included an anti-α1 antibody (TS2/7) and an anti-α2 antibody (P1H5). In total 5 mM and 25 mM glucose exposed cell extracts were immunoprecipitated side by side 3 times.

The α1 subunit band was clearly discernible at 180 kD in cell samples exposed to 5 mM of glucose and was associated with a β1 band (116 kD). No α1 band could be seen in the 25 mM treated cell sample. In contrast, the α2 subunit band was more prominent in cell samples exposed to 25 mM glucose and appeared as a band at 130 kD. The 130 kD α2 band was present in 5 mM glucose but was significantly less intense than the 25 mM glucose treated samples.

The cell lysates were also incubated with the following antibodies including: SP2 myeloma culture supernatant; anti-β1 (P5D2), anti-β2 (P4H9), anti-α2 (P1H5), anti-α3 (P3D11), anti-α4 (P4G9), anti-α5 (P3D10) and anti-α6 (G0H3). Results were interpreted from three independent experiments. Immunoprecipitation of α3-α6 and β1 integrin subunits was performed on cells from the two growth conditions. Subunits α4 and α6 were not detected in either cell population. The antibody to the β1 subunit precipitated a 116 kD protein, the β1 subunit, and also a precursor β1 band at 105 kD. The α3 and α5 subunits were seen at $\approx$ 130 kD with the associated β subunit at 116 kD, in both cell populations.

<u>Flow cytometry</u>

Cell surface expression of integrin subunits by cultured human mesangial cells was evaluated by indirect immunofluorescence staining and flow cytometry. Mesangial cells were released with trypsin, washed and resuspended in FACS buffer (HBSS, 2% goat serum, 0.02% sodium azide). An equal number of cells, 2 x 10^5 were added to each vial.

The cells were incubated with primary antibody for one hour at 4°C and washed once with 1 ml FACS buffer. The secondary antibody was then added in a total volume of 0.5 ml FACS buffer and incubated for 30 minutes at 4°C. The cells were again washed in 1 ml of FACS buffer and resuspended in 0.5 ml of 2% formaldehyde.

The data was analyzed using CONSORT 30 software on a FACScan (Becton Dickinson, Mountain View, CA). Positive fluorescence was determined on a four decade log scale and fluorescence (log F1) was expressed as the mean channel number of 5,000 cells. Cell surface expression experiments were performed in duplicate with each antibody, at least three times with each growth condition of mesangial cells.

Densitometric scanning of the fluorograms generated from metabolically labeled cells indicated that the synthesis of the β1 (12%), α3 (14%) and α5 (19%) were moderately increased upon growth in 25 mM glucose. Growth in 25 mM glucose dramatically decreased synthesis of the α1 subunit (39% reduction in intensity) while synthesis of α2 was considerably increased (42%).

These changes in metabolic activity were paralleled by a similar change in the cell surface integrin phenotype of mesangial cells grown in high glucose. To assess the effect of different glucose concentrations in the medium on the levels of mesangial cell surface integrin receptor expression cells in each glucose treatment population were stained for immunofluorescence and processed for flow cytommetry. Mean channel fluorescence (MCN) values of integrin subunit expression were obtained from 3 experiments. Within each experiment the ratio of MCN for cells grown in high glucose (HG) to cells grown in low glucose (LG), denominator = 1 was calculated.

Cell surface expression of the following integrin subunits was increased by growth in high glucose: β1 (24%), α2 (26%), α3 (18%), and α5 (19%). The decrease in the synthesis of α1 was reflected in a concomitant decrease in cell surface expression (33% reduction in specific staining). The α4 and α6 subunits were not detectable in cultured mesangial cells either by immunoprecipitation or flow cytometric analyses.

Mesangial cells grown in high glucose (for at least 2 passages) were returned to control media (5 mM glucose), again for at least 2 passages. A flow cytometric analysis of these cells revealed a reversion to "low glucose" type. The expression of α2, α3, α5 and β1 were decreased while the expression of α1 increased (data not shown).

Example 2
Adhesion of Cultured Mesangial Cells to
Type IV Collagen (cIV): Effect of High Glucose

Cell adhesion to collagen IV (cIV)

The cells were detached from culture flasks by incubation with trypsin 0.05% and EDTA 0.02% for two minutes at 37°C, then washed twice with DMEM and resuspended to the appropriate concentration in binding buffer (DMEM, 25 mM HEPES, 2 mg/ml BSA at pH 7.4). 48 or 96 well plates were coated overnight at 29°C with cIV in serial dilutions starting from 100 μg/ml (5 μg/96 well or 20 μg/48 well). Under these conditions approximately 50% of the cIV adhered. To block the remaining reactive sites the plates were treated with 200 μl of BSA at 2 mg/ml for 2 hours at 37°C. 50 μl of suspension containing 5000 cells (96 well plates) or 100,000 cells (48 well plates) was added per well. The plates were incubated at 37°C in a humidified incubator for approximately 30 minutes. The non-adherent cells were removed by washing three times with binding buffer and then 100 μl of "lysis" buffer (0.5 NaOH, 1% SDS in distilled water) was added to each well for 30 minutes at 60°C. The lysate was transferred to scintillation vials and counted. The data was expressed as a percentage of the total input cpm. Cell adhesion assays were performed in triplicate, at least three times for each growth condition.

Cells grown in medium containing 25 mM glucose adhered significantly better than cells in 5 mM glucose. Adhesion increased with coating concentration of cIV and was saturated at 25 μg/ml for both cell populations.

Inhibition of cell adhesion with monoclonal antibodies

Since growth in high glucose appeared to alter the synthesis and expression of the integrin receptors α1β1 and α2β1 which have been reported to be involved in cell adhesion to collagen, (Wayner and Carter, *J. Cell. Biol.* 105:1873 (1987)), we examined the effects of glucose on the ability of mesangial cells to adhere to cIV.

Monoclonal antibody inhibition of ^{35}S-methionine labeled human mesangial cells grown in 5 mM glucose to cIV was assessed. Briefly, 96 or 48 well plates were coated with 50 or 200 μl of cIV at 2.5 μg/ml, overnight at 29°C. The plates were incubated with 2% BSA in PBS to coat remaining reactive sites on plastic for 2 hours,

and then hybridoma culture supernatant or ascites containing 10 μg/ml of antibody were added to each well, followed immediately by the cells. After 30 minutes non-adherent cells were washed off and adherent cells were quantitated. Results were obtained from 3 experiments. SP2 myeloma culture supernatant of W6/32 were used as negative controls. A quantitative ELISA was used to determine the concentration of antibody in the hybridoma culture supernatant or ascites.

In each case, the concentration of monoclonal antibody (Mab) was determined relative to a standard curve generated with an isotype-matched control mouse IgG. The concentration of antibody required to saturate the binding sites on human mesangial cells was determined by flow cytometry. The concentration of the antibodies used in the inhibition assays were well above the saturating concentration as determined by flow cytometry. Data were expressed as the percent of maximal binding observed in the presence of W6/32 antibody. Inhibition experiments were performed at least three times, in triplicate, for each growth condition with the various antibodies.

Mesangial cells grown in high glucose (25 mM) adhered better to cIV than cells grown in low glucose (5 mM). Results indicated that adhesion increased with coating concentration of collagen IV and saturated at about 25 μg/ml for both cell populations.

In order to examine the activity of collagen receptors expressed by mesangial cells grown in high glucose, we performed adhesion experiments in the presence of well characterized neutralizing antibodies directed to various β1 integrin subunits. A panel of antibodies was used all of which have been reported to inhibit the adhesion of cells to various substrates (Wayner and Carter, cited *supra*, 1987; Wayner et al., cited *supra*, 1993). Antibodies were used at saturating concentrations as determined by immunofluorescence staining and flow cytometry. In the competition experiments, the following criteria were selected to promote half-maximal binding of mesangial cells: 2.5 μg/ml cIV and a short term assay (less than 30 min). The ability of neutralizing Mabs to inhibit mesangial cell adhesion to cIV was examined in low (5 mM) or high glucose (25 mM) containing media.

To test Mab-mediated adhesion inhibition of mesangial cells grown in 5 mM glucose or 25 mM glucose to collagen IV, ^{35}S-methionine labeled human mesangial cells were seeded in 48 well plates (100,000 cells/well) coated with 200 μl cIV (2.5 μg/ml, overnight at 29°C). Mab's anti-α1, SR84, anti-α2, P1H5, anti-β1, P5D2 and

SR84 and P1H5 together, were added to the wells before seeding with cells. Adhesion in the presence of W6/32 was used as a control. After 20 minutes non-adherent cells were washed out and adherent cells quantitated. The data was expressed as a percentage of the binding in the presence of W6/32. and the two cell populations were normalized by using the binding in the presence of HLA antibody to represent 100% and the inhibition by other antibodies was calculated as a percentage of binding in the presence of HLA.

The results indicated that the $\alpha1\beta1$ integrin receptor had a reduced role (*$p < 0.001$) for cells grown in 5 mM glucose as compared with 25 mM glucose. Of the antibodies examined, only Mabs directed to the $\alpha1$ (SR84), $\alpha2$ (P1H5) or $\beta1$ (P5D2) integrin subunits inhibited the binding of mesangial cells to cIV. When mesangial cells were grown in either low or high glucose, adhesion to cIV could be almost completely inhibited with Mabs to $\beta1$ (P5D2) or a combination of $\alpha1$ (SR84) and $\alpha2$ (P1H5).

The relative effects of the neutralizing Mabs directed against the $\alpha1$ and $\alpha2$ subunits varied depending on whether mesangial cells were grown in low or high glucose. In 5 mM glucose the Mab to the $\alpha1$ subunit of integrins resulted in more inhibition ($\approx$ 50%) than in 25 mM glucose ($\approx$ 20%) ($p < 0.001$). This is consistent with the presence of significantly more $\alpha1$ integrin on the surface of cells grown in 5 mM glucose. Alternatively, in 5 mM glucose the Mab to the $\alpha2$ subunit resulted in less inhibition ($\approx$ 60%) than in 25 mM glucose ($\approx$ 75%) ($p < 0.001$). Mab's against the $\alpha3$, $\alpha4$, $\alpha5$ and $\alpha6$ subunits did not inhibit adhesion (data not shown).

These data demonstrate that under low glucose growth conditions, mesangial cells use $\alpha1\beta1$ and $\alpha2\beta1$ integrins to bind cIV coated surfaces. However, cells grown in high glucose, appear to rely more on the $\alpha2$ subunit complexed with $\beta1$. The results of these functional studies are consistent with the observed alterations in the integrin cell surface phenotype discussed in Example 1.

Example 3
Localization of $\alpha1\beta1$ and $\alpha2\beta1$ Integrin Receptors
Localization of $\alpha1$ integrin in focal adhesions

Glass cover slips were coated with 50 µl of cIV at 2.5 µg/ml, overnight at 29°C. The coated areas were "blocked" for two hours with BSA at 2 mg/ml, in PBS. Human

mesangial cells were processed as before, seeded on each spot of cIV in 50 μl of binding buffer (2500 cells) and allowed to adhere for 5 hours at 37°C. The unbound cells were washed off with PBS. Adherent cells were fixed with 2% paraformaldehyde in HBSS for 30 minutes followed by permeabilization with 0.5% Triton X-100 for 2 minutes. The cells were blocked again with PBS following which 200 μl of hybridoma culture supernatant containing anti-α1 antibody (TS1/7) was added to each spot and incubated at room temperature for 1 hour. The coverslips were then thoroughly washed and rhodamine-conjugated goat anti-mouse antibody (1:100) (Boehringer Mannheim, Indianapolis, IN) was added for one hour. The coverslips were again washed and incubated with anti-vinculin antibodies (Sigma, St. Louis, MO) preconjugated (Quicktag, FITC labeling kit, Boehringer Mannheim, Indianapolis, IN) to FITC labeled goat anti-mouse antibody for 1 hour at room temperature. The coverslips were finally washed, mounted on glass slides and viewed for focal adhesions by co-localization of vinculin with α1 integrin.

<u>Staining of normal human adult kidneys for the presence of β1 integrins</u>

Normal human adult kidney tissue was snap frozen in liquid nitrogen and sections were prepared with a cryostat at 5 μm intervals. The sections were stained using an anti-mouse Vectastain Elite Kit (as described by Wayner et al., 1993) with diamino benzene (DAB) as the chromogen. The following mAbs were used: α1 (TS2/7), α2 (P1H5), α3 (P3D11), α4 (P4G9) and β1 (P5D2). These monoclonal antibodies are available from the following sources and stained the following histological areas as was demonstrated in these studies:

α1 (TS2/7)	Martin Hemler, Dana Farber Cancer Center, Boston, MA. Stained mesangium.
α2 (P1H5)	EA Wayner, Fred Hutchinson Cancer Center, Seattle, WA Stained mesangium.
α3 (P3D11)	EA Wayner, Fred Hutchinson Cancer Center, Seattle, WA Stained the mesangium, endothelium, visceral and Bowman's epithelium and capsule.
α4 (P4G9)	EA Wayner, Fred Hutchinson Cancer Center, Seattle, WA Did not stain glomeruli.

β1 (P5D2) EA Wayner, Fred Hutchinson Cancer Center, Seattle, WA Stained mesangium, endothelium, visceral epithelium, Bownman's epithelium and capsule.

Normal mouse IgG (all isotypes) was used as a negative control.

These studies demonstrated the presence of α1β1 and α2β1 integrin receptors in focal adhesions. Focal adhesions are observed when cells spread in culture on matrix components such as collagen IV, fibronectin or laminin. Integrins cluster at the site of focal adhesions on the cell surface with intracellular fibers such as vinculin staining at these locations within the cell periphery. (see Hynes, et al. *Cell* 69:11-25, 1992 and Burridge, et al. *Ann. Rev. Cell Biol.* 4:487-525, 1988). This supports the hypothesis that mesangial cells use α1β1 and α2β1 integrin receptors to bind to cIV. It has been well established that when a particular integrin receptor is engaged by a specific ligand it can be detected in focal contacts co-localized with certain components of the cytoskeleton such as vinculin. Therefore, we asked whether mesangial cells could localize α1 (or α2 and β1) to focal adhesions when seeded on cIV coated substrates.

α2 or β1 could be detected in focal contacts on cIV regardless of whether mesangial cells were grown in either low or high glucose. Additionally, when mesangial cells were grown in 5 mM glucose and subsequently seeded on cIV coated surfaces, α1 could also be co-localized with vinculin within several focal contacts by dual-label immunofluorescence staining. It is believed that cIV binding in cells maintained in low glucose engages both the α1 and α2 subunits. α1 could be detected in only some of the focal adhesions stained by vinculin. As a control, α1 was not detected in focal contacts when mesangial cells were seeded onto fibronectin coated surfaces regardless of the glucose concentration of the cell culture media.

Immunohistochemical staining of integrin receptor subunits in normal human adult and fetal kidney revealed that both α1 and α2 could be localized within the mesangium. The α1 receptor was diffusely expressed throughout the mesangium whereas the distribution of α2 was more limited and focal. Also consistent with the results we obtained with cultured mesangial cells, β1 and α3 were intensely expressed throughout the mesangium, while α4 could not be detected in either fetal or adult mesangium.

Example 4
Alterations in RNA Production in Human Mesangial Cells cultured in High and Low Glucose Concentrations

Our efforts have concentrated on finding a way to predict, at early stages after the onset of diabetes, the subjects who will later develop nephropathy. We focused on a major hallmark of diabetic nephropathy, that of mesangial expansion. We first examined mesangial cells in culture, since these cells secrete their surrounding matrix, which is expanded in diabetes; however, biopsied tissue can be treated in the same manner, as will be understood by those skilled in the art. The matrix consists primarily of collagen IV.

Primary cultures of human mesangial cells undergo several phenotypic changes in response to elevated glucose concentrations and glucose-modified ("glycated") collagen IV. These changes included altered cell interactions with the collagen matrix. In elevated glucose concentrations, the $\alpha 1$ subunit underwent a substantial decrease, concomitant with an increase of the $\alpha 2$ integrin subunit. This change was observed with immunoprecipitation and flow cytometry. Further studies with Northern analysis and *in situ* hybridization of the cultured mesangial cells confirmed the integrin reversal. In the studies employing Northern analyses, separate samples of total RNA were isolated from the mesangial cells on each culture plate or alternatively from rat kidneys (see Example 5, below) by a single-step method using RNA STAT-60™ isolation reagent (TEL-TEST "B", INC., Friendswood, TX) according to the manufacturers directions. Briefly, the cells were lysed with RNA STAT-60™ solution by repetitive pipetting; the tissues were cut into small pieces and homogenized in the RNA STAT-60 solution with a high-speed tissue homogenizer (Polytron CH6005, Luzern, Switzerland). The nucleic acid mixture was extracted with 0.2 ml chloroform per 1ml of the RNA STAT-60™ solution. Total RNA was precipitated for 10 min at -80°C in isopropanol, and the pelleted RNA was redissolved in TE buffer. The total RNA was free of DNA and proteins and had a 260/280 wavelength ratio > 1.8.

Northern blot analysis-The RNA samples were denatured in formaldehyde gel-running buffer (20 mM MOPS, 8 mM sodium acetate, mM EDTA, at pH 7.0) containing 6% formaldehyde and 50% formamide by heating at 65°C for 15 min. For each sample 20 mg of RNA was mixed with 6x loading buffer (50% glycerol, 1 mM

EDTA, 0.25% bromphenol blue, 0.25% Xylene cyanol FF), loaded on a 1% agarose gel submerged in 6% formaldehyde running buffer, and run at 3-5 V/cm for 3-4 hours. RNA was transferred from the agarose gel to a nylon membrane (Boehringer Mannheim, Indianapolis, IN) by capillary elution and immobilized by UV cross-linking (Stratalinker UV; Stratagene, La Jolla, CA). The membranes were then incubated in prehybridization solution containing 50% formamide, 5xSSC. 0.02% SDS, 0.1% N-lauroylsarcosine, 2% blocking reagent (Boehringer Mannheim), and 20 mM sodium maleate (pH 7.5) for >3 hours at 42°C. Radiolabeled probes (see Example 5) for the integrin subunits or controls were then added to the prehybridization solution and hybridization was performed overnight at 42°C (for cDNA probe) or 50°C (for antisense RNA probe). After hybridization, the membranes were initially washed in 2x SSC, 0.05% SDS for 10 minutes at room temperature and then washed for an additional 40 minutes at 42°C (for cDNA probe) or 60°C (for antisense RNA probe). Membranes were then exposed to X-ray film (X-Omat RP; Eastman Kodak Co., Rochester, NY) for 1 day at -80°C. After being stripped of previous probes by heating in 0.2x SSC, 0.5% SDS for 10 min at 100°C, the membranes were reprobed as described above. Images of autoradiograms were captured and digitized using a CCD video camera module interfaced with a microcomputer (Macintosh IIcx: Apple Computers Inc., Cupertino, CA) and analyzed using image processing software (NIH Image 1.55b77: public domain).

Cells grown in 25 mM glucose expressed lower levels of $\alpha 1$ integrin than seen in an equivalent amount of RNA from cells grown in 5 mM glucose. Densitometric analysis demonstrated an ≈30% decrease upon averaging the values from four samples. Similar analysis demonstrated ≈30% increase in $\alpha 2$ integrin expression in cells grown in 25 mM glucose.

Example 5
In Situ Hybridization Detecting Expression of Integrins in Kidney Sections Taken at Various Times After Onset of Diabetes

The expression of $\alpha 1$ and $\alpha 2$ integrin receptors was examined in rat kidney sections after the onset of diabetes.

The *in situ* hybridization approach was used to examine kidney sections of streptozotocin-diabetic rats, 2.5 months after induction of diabetes. At this time interval, glomerular changes were still minimal. The streptozotocin-induced diabetic rat model mimics human changes of mesangial expansion and glomerular basement membrane thickening in late nephropathy and is an art accepted model for diabetes and nephropathy.

Female non-pregnant Sprague-Dawley rats were obtained from Brithwood, Minneapolis, MN. The animals weighed 190-210 g at the beginning of the experiments and were given a 52mg/kg intraperitoneal dose of streptozotocin (STZ, Zanazar brand, Upjohn Corp., Kalamazoo, M1) in calcium citrate and calcium carbonate Buffer (pH 4.5) to induce diabetes, while the controls were injected with the same amount of Hanks' balanced salt solution (pH 7.2). The animals were fed on standard rat chow (Purina laboratory chow # 5001. RFG PET@Supply Company, Plymouth, MN), and tap water *ad libitum*. Presence of diabetes was confirmed by detection of >400mg/dl nonfasting plasma glucose levels 10 days post injection by tail vein bleeding using the glucose peroxide method (Beckman glucose analyzer, Beckman Instruments, Inc., Fullerton, CA).

Body weight was determined weekly, blood glucose levels were determined at 4 weeks after induction of diabetes, and on the day before the termination of the experiment, which was 2.5 month from induction of diabetes. Urinary albumin excretion (UAE) was determined by radial immunodiffusion Mancini method, using goat IgG fraction against rat albumin (Cappel Cat. No. 55727) and purified rat albumin (Cappel Cat. No. 55952, Cappel Research Products, Durham, NC), according to previously published procedures (Mauer et al, *Diabetes* 27:959-64, 1978). Rats were sacrificed at 2.5 months after diabetes induction and kidney tissue was perfusionally fixed by injecting freshly prepared 4% paraformaldehyde through the renal artery. This was followed by overnight fixation in 4% paraformaldehyde after removal from the body. The tissue was sectioned at 5 μm and placed on the silane-coated slides (Digene Diagnostics, Inc., Beltsville, MD) for *in situ* hybridization with probes for the α1 and α2 integrin subunits.

2.5 months after injection of STZ, diabetic rats weighted significantly less than controls, whereas their right kidney weight and serum glucose concentration were

significantly increased, as compared to the controls (see Table 1). Diabetic and non-diabetic rats demonstrated no significant difference in glomerular size and albumin excretion at 2.5 month after induction of diabetes (Table 1).

TABLE 1

TISSUE	CONTROL	DIABETIC	S/NS
Body Wt.(g)	390+/-10	200+/-20	S
Right Kidney wt. (g)	1.35+/-0.1	1.8+/-0.1	S
Plasma glucose (mg/dl)	140+/-25	760+/150	S
Glomerular area	1.42+/-0.5	1.45+/-0.6	NS

A 5.4 kb human α2 integrin CDNA clone (Takada, et al., 1989, *supra*) and a rat αl integrin cDNA clone (Ignatius et al, *supra*) in bluescript vector (Stratagene, La Jolla, CA) were used in these experiments. A 1.79 kb α2 integrin cDNA fragment was restriction digested from the EcoRI site. Similarly, a 3.98 kb α1 integrin cDNA fragment was obtained by restriction digestion from the EcoRI site.

cDNA fragments were purified by GENE CLEAN II kit (BIO 101, San Diego, CA) and labeled using the random primer labeling kit (Boehringer Mannheim, Indianapolis, IN) with P^{32}-dCTP (NEN) for Northern blotting and with S^{35}-dCTP (NEN) for *in situ* hybridization. GAPDH and sheep visna virus cDNA (PLV-KS) (Staskus et al, *Virology* 181:228-240, 1991) probes were used as the positive and negative controls respectively.. The probes preferably had a specific activity of 2×10^8 - 1×10^9 dpm/μg.

By Northern blotting, compared to the controls, the diabetic kidneys expressed 113.5% more α1(IV) RNA, 46.5% more α3(IV) RNA, 54.8% less metalloproteinase-2 RNA (MMP-2, an enzyme that cleaves type IV collagen) and 246% more TIMP-1 RNA (a tissue inhibitor of metalloproteinases) with a $p < 0.01$ in all cases as determined by ANOVA.

The expression of αl and α2 integrin RNA was localized using a modification of a previously described method for *in situ* hybridization (Staskus et al. *supra*). 5μm tissue sections on silane-coated slides were fixed in the freshly prepared 4% paraformaldehyde for 10 min. The slides were pretreated with 0.2N HCl for 20 min, 0. 15 M Triethanolamine (TEA, Sigma, St. Louis, MO) for 15 min, 0.005% digitonin for 5 min, 3 mg/ml proteinase K (Sigma) for 15 min at 37°C, and O.3% acetic anhydride -

O.1M TEA for 10 min. Hybridizations were performed under stringent hybridization conditions. Stringent hybridization conditions are defined in this specification as 50°C overnight, in 50% formamide, 0.6 M NaCl, 1x Denhardt's solution, 0. 17 mg/ml human COT^{RT} DNA (GIBCO/BRL), 1 mg/ml poly A (Boehringer Mannhieim), 10% (w/v) Dextran sulfate (Sigma), 0. 1 M dithiothreitol (DTT, Boehringer Mannheim), 1 mM EDTA, 0. 1 mM aurinitricarboxylic acid (ATA, Sigma) and S^{35}-dCTP labeled cDNA probe. The next day, the slides were washed in 2x SSC-0.05% SDS for 60 min at 55°C (recipes for SSC and the like can be found in Sambrook, et al., *supra*); further washed in a high stringency washing buffer containing 50% formamide, 0.6 M NaCl, 1 mM EDTA, 5 mM DTT and 10 mM Hepes for 4 days at room temperature. After a brief rinse in 2x SSC, the slides were dehydrated in graded ethanol with 0.3 M ammonium acetate then dipped in Kodak NTB-2 emulsion and exposed for 5 days at 4°C.

After development the slides were stained with hematoxylin-eosin (Surgipath Canada, 1nc., Winnipeg, Canada) and mounted. A ratio of the number of silver grains per cell was used to quantitate the results of *in situ* hybridization. Twenty glomeruli each were counted from each control and diabetic animal. Each glomerulus was assessed for: 1) glomerular area; 2) glomerular perimeter; 3) grains per glomerulus; and 4) number of cell nuclei per glomerulus.

The results were estimated as grains per cell nucleus and grains per glomerular area, as mean +/- SD of 5 animals (20 glomeruli each). (Haase, A.T., [1990]: *In situ* hybridization, CRC Press, 199-217; Nuovo, G.J., [1992] PCR *in situ* hybridization, protocols and applications, Raven Press). Groups were compared with the 2-tailed student t-test. Differences between groups were considered significant at $p<0.05$.

The results are illustrated in Fig. 1. Early after induction of experimental diabetes, the expression of the $\alpha 1$ integrin subunit by glomerular cells was decreased compared to the control, whereas the expression of $\alpha 2$ integrin was increased. The average counts, in diabetic glomeruli hybridized with the $\alpha 1$ integrin probe, were significantly lower than control (Fig. 1). Also, the average counts, in diabetic glomeruli hybridized with the $\alpha 2$ integrin probe, were significantly higher than control (Fig. 1).

Control animals at 2.5 month diabetes expressed on an average a significantly higher level of $\alpha 1$ subunit integrin and significantly lower levels of $\alpha 2$ subunit integrin using unbiased methods of selection of areas for study. The entire section was surveyed

for RNA grains, the regions of the Bowman's space and the background count were excluded by studying a commensurate area of the negative control stained tissue.

Compared to the control, glomerular cells (GC:endothelial, epithelial and mesangial combine) and/or tubular (proximal and distal epithelial) cells (TC) had 36% (GC) less grains for $\alpha 1$ integrin; 86.4% (GC) more grains for $\alpha 2$ integrin; 82(TC)-167% (GC) more grains for $\alpha 1$(IV); 107 (TC)-137% (GC) more grains for $\alpha 3$(IV); 63.6(GC)-65.3%(TC) less MMP-2.

The results of the present study clearly demonstrate that mesangial cells, when cultured in high glucose (25 mM) instead of normal/low glucose (5 mM) alter their RNA production for the integrin subunits $\alpha 1$ and $\alpha 2$. Thus, this phenomenon is observed both at the level of protein and RNA production.

Furthermore, the results of our *in situ* hybridization and immunohistochemical staining experiments show that these changes can be detected in the mesangium of diabetic rat kidney and that human $\alpha 2$ integrin subunit probes and rat $\alpha 1$ integrin subunit probes are functional in both rat and human cells. Work by Mendrick and co-workers (*Lab. Invest.* 72(3):367-375, 1995) has shown that in the rat both integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ of mesangial cells interact with collagen; as happens in the human mesangial cells. In the present study, the distribution of $\alpha 1$ and $\alpha 2$ integrin receptor subunit RNA was precisely localized by *in situ* hybridization to the different cell types of the glomerulus and surrounding tubules. Normal rat tissues expressed levels of the $\alpha 1$ subunit and also the $\alpha 2$ subunit RNA, as determined by counting the number ratio of silver grains/cell. However, the streptozotocin-induced diabetic animals had significantly lower levels of RNA for the $\alpha 1$ subunit and significantly higher levels of $\alpha 2$ subunit. A similar distribution of $\alpha 1$ and $\alpha 2$ subunit RNA (silver grains) was seen in the proximal and distal tubular epithelial cells. These data indicate that the distribution of cell surface integrin expression may be regulated by gene expression at the transcriptional level.

In summary, using *in situ* hybridization, similar results were seen in both mesangial cells *in vitro* and in glomeruli from tissue sections probed for the $\alpha 1$ and $\alpha 2$ integrin.

Early after induction of streptozotocin-diabetes in rates, substantial matrix-related gene expression changes occurred. For example, $\alpha 1$ and $\alpha 2$ integrin levels

changes, components of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrin cell receptors for tIV (an important component of the renal extracellular matrix) underwent a reversal in levels with less $\alpha 1$ and more $\alpha 2$ integrin being present in glomeruli from kidneys of diabetic rats, when compared to the control. Expression of tIV was increased whereas the expression of MMP-2 which degrades tIV was substantially decreased. TIMP-1, an inhibitor of MMP-2 was increased. The observed matrix changes indicate an imbalance of tIV synthesis and turnover. This dysmetabolism of tIV, apparent in both the glomerular and tubular areas of the kidney, occurred before significant renal functional changes, or matrix accumulation out of proportion to renal enlargement, could be detectable. These changes could have a regulatory role in significant basement membrane thickening and mesangial expansion of diabetic nephropathy.

Collectively, the obtained data indicate that increased glucose concentration induces quantitative changes in receptor synthesis and cell surface integrin expression of human mesangial cells. In the diabetic, all cell systems are exposed to hyperglycemia and it is know that many cell and organ systems are affected by the disease; therefore, other cell types could similarly be used to assess changes in the levels of $\alpha 1$ and/or $\alpha 2$ integrin subunit expression as a measure of a predisposition to a variety of diabetic-induced pathologies. Kyu-Jin, et al. (*supra*) have noted alterations in integrin subunit expression in skin fibroblasts of diabetic patients. This information, in conjunction with the data discussed herein, indicates that altered levels of integrin subunit expression can be detected from a variety of integrin-expressing cells in diabetic nephropathy patients.

These results support the *in vitro* primary human mesangial cell culture data demonstrating that changes in cell surface integrin expression indicate the onset of nephropathic changes.

Example 6
Detection of Altered Levels of α1 and α2 Integrin Subunit Expression in Humans using Blood and Tissue Samples

Patients with insulin-dependent diabetes mellitus (IDDM), individuals at risk for developing IDDM, patients with clinical diabetes nephropathy and healthy age matched volunteers are selected for studies to confirm the presence of altered $\alpha 1$ and $\alpha 2$ integrin subunit expression in integrin-producing cells. Clinical diabetic nephropathy is defined by the presence of persistent proteinuria (urinary AER > 300 μg/day) in sterile urine of

patients with >10 yr duration of disease and concomitant retinopathy and is confirmed by the presence of classic glomerulosclerotic lesions on renal biopsy. Normal, nondiabetic individuals without a family history of hypertension serve as control subjects.

Patients were biopsied as follows: For skin biopsies, a biopsy is taken from the anterior surface of the left forearm by excision under local anaesthetic such as ethyl chloride, see Trevisan, et al. *Diabetes* 41:1239-45, 1992. The biopsy is optionally divided in half. With half of the tissue frozen immediately in liquid nitrogen and the other half placed in Hanks balanced salt solution. The frozen tissue is embedded in paraffin and processed for *in situ* hybridization as has been described above. A portion of the intact tissue is preferably immediately minced and processed for RNA isolation using techniques described above. Remaining minced tissue is gently digested with trypsin to obtain a cell suspension, washed in media containing serum to remove trypsin and plated onto tissue culture dishes containing 10% FCS supplemented DMEM with antibiotics.

Renal biopsies were obtained as follows. Patients should have normal blood pressures, normal coagulation values and platelet counts. Ultrasound was used to precisely localize the kidney. Ultrasound was also used to determine renal size, structural defects and post-void residual urine. Renal biopsies were performed on sedated patients using the Franklin modified Vim-Silverman or Truecut needles available from surgical supply suppliers. The biopsy specimens were immediately examined under a dissecting microscope to ensure that adequate samples of glomeruli were present for subsequent studies to quantitate integrin levels. Biopsied tissue was sectioned and processed for *in situ* hybridization as described in Example 5. In one example, renal samples from diabetic patients who did not show signs of microalbuminuria, but who had diabetic siblings with renal nephropathy were processed for *in situ* hybridization and PCR *in situ* hybridization. Renal samples from diabetic patients without a family history of nephropathy were also studied by PCR *in situ* hybridization to detect altered levels of integrin subunit expression.

PCR *in situ* hybridization is performed as follows. Sections are fixed as described in Example 5 and rinsed in RNase free water. The protocol used is that described by Nuovo, et al. (*Am. J. Surg. Pathol.* 17:683-690, 1993.) Cells are treated

with pepsin and DNase as described. cDNA synthesis is initiated by adding 10µl of a solution containing one or more of the following probes listed in a 5'-3' orientation with their SEQ ID NOS and their nucleic acid location on the respective integrin gene with reverse transcriptase (Perkin-ELmer, Norwalk, Conn.):

α1 integrin primer	SEQ ID NO	NA location
CCAGAGTCACTCTCACAGAG	5	2729-2748
CACAGCGTACACGTACACC	6	1991-2009
CACTTATAGACATCTCCAG	7	646-664

α2 integrin primer	SEQ ID NO	NA location
CATCCATGTTGATGTCTG	8	1733-1750
CATGTGATTCACCGTCAG	9	894-910
GCATATTGAATTGCTCCGAATGTG	10	801-826

The resulting cDNAs are subjected to amplification containing a 1 µM concentration (each) of one or more of the above primers with a paired primer located 5' to the primers provided above. Those skilled in the art will recognize that a variety of other primers could also be used from the α1 and α2 integrin gene sequence to similarly perform PCR *in situ* hybridization. The preferred primers paired with the above primers are provided below.

α1 integrin primer	SEQ ID NO	NA location	SEQ ID Pair
GGCGTATGCACAACGCA	11	2261-2277	5
GCGACAGCTGACCAGTCAGCA	12	1509-1529	6
CACTCCTCCACAGCTCCT	13	251-268	7

α2 integrin primer	SEQ ID NO	NA location	SEQ ID Pair
ACATGTACTCACTGG	14	1593-1608	8
CTCACATGTGGTCCTCTG	15	433-451	9
GTCCTGTTGACCTATCCACTGC	16	296-319	10

The SEQ ID Pair in the above table refers to the paired primer that provides amplification of the sequence positioned between the primer pairs on the respective integrin gene. The PCR products are detected by using an antidigoxigenin-alkaline phosphatase conjugate and the chromagen nitroblue tetrazolium (NBT)-5-bromo-4-chloro-3-indoylphosphate toluidinium (Salt) (BCIP). The counterstain nuclear fast red

is used to stain nuclei. Internal probes located within the nucleic acid regions amplified by PCR can also be used to identify the amplified fragments. Thus, based on the pairings provided above, oligonucleotide probes can be selected between regions 267-645, 1530-1990 and between 2278-2728 for the α1 integrin gene and between regions 320-800, 452-893, 1607-1732 for the α2 integrin gene and hybridized and stained following the *in situ* hybridization methods detailed in Example 5.

A blood sample is also taken from the patient and leukocytes are isolated from blood by centrifugation, followed by hypotonic shock of residual blood cells. The leukocytes are then processed for *in situ* hybridization as has been discussed in the preceding examples.

Results:

PCR *in situ* hybridization with renal tissues demonstrated decreased α1 and increased α2 integrin subunits in the patient with diabetic neuropathy as compared with control tissue.

Quantitative analysis of RNA grains per unit area of kidney glomeruli and tubules was performed by counting silver grains under epi-polarized light.

As shown in Table 2, both glomeruli and tubules of the diabetic neuropathy patient showed significantly decreased α1 integrin levels as compared to the control, whereas α2 integrin levels were significantly increased as compared with control levels.

TABLE 2

	Glomeruli[a]		Tubules[a]	
Sample	α1	α2	α1	α2
Control	156	83	136	101
Diabetic Neuropathy	121[b]	95[c]	89[c]	124[b]

[a] = grains per unit area [b] = $p < 0.05$ [c] = $p < 0.01$

These results confirm the *in vitro* observations in mesangial cells that there is a decrease of the α1 integrin subunit and a concommitant increase of α2 integrin

expression in a diabetic nephropathy. This represents a reversal of mesangial integrins which mediate binding of mesangial cells to collagen IV.

Example 7
Increased Integrin Subunit Expression in Skin Fibroblasts From Diabetic Patients with Nephropathy as Compared with Control Diabetic Patients

Fibroblasts were obtained from skin biopsies from diabetic patients with or without diabetic nephropathy and cultured as described for Example 6. Expression of α3, α5, and beta-1 integrin subunits in the cultured cells was analyzed by Northern blotting and subsequent densitometry, as described above, and using published probes.

For the α3 integrin subunit, the 1.9 SalI fragment described in Takada Y., et al., *J. Cell Biol.* 115:257-266 was used. For the β1 subunit, the 3.6 kb insert of the β1 subunit (the whole cDNA), described in Giancotti and Ruoslahti, *Cell* 60:849-850 (1990) was used. For the α5 subunit, the 3.7 kb SalI-Xba insert of the α5 subunit (the whole cDNA) described in Giancotti and Ruoslahti, *Supra* as used. These probes were radiolabeled and used under the same conditions as those described for Example 6.

The study included five patients per group, five each from the normal, diabetic "slow track" and from the Diabetic "fast track". Both groups of diabetic human subjects had renal function studies and kidney biopsies performed as part of their evaluation as possible candidates for pancreas transplantation. All procedures were approved by the Committee on Human Subjects at the University of Minnesota, and all patients gave written consent. All patients spent one week at the Clinical Research Center (CRC) at the University of Minnesota for pre-pancreas transplant evaluation, during which time they underwent multiple 24-hour urine collections (at least three) for measurements of creatinine clearance and urinary albumin excretion. Blood pressure was measured repeatedly by the CRC nursing staff. HbA1 was used to assess glycemic control. All patients underwent percutaneous kidney biopsy and skin biopsy. Patients were divided into two groups based on criteria of severity of renal lesions determined by morphometric analysis of mesangial functional volume and IDDM duration.

"Normal" samples were kidney biopsies from non-diabetic human subjects, taken to examine for the presence of neoplastic tissue, etc., on which a similar analysis to that performed for the diabetic tissues was done. These subjects underwent similar

renal functional studies to make certain that albuminuria, increased creatinine clearance, or hypertension were not present.

The data, shown below in Table 3, demonstrate a significant increase in $\alpha3$ and beta-1 subunit expression in the skin fibroblasts of diabetic nephropathy patients as compared with the control diabetic patients.

TABLE 3

Integrin Subunit	Normal Values	Control Diabetic	Nephropathy Diabetics	p
$\alpha3$	11.5 (9.1-13.3)	10.1 (8.6-12.8)	17.1 (16.1-35.6)	<0.5
$\alpha5$	36.2 (18.3-46.6)	38.7 (31.6-57.2)	30.3 (13.2-48.4)	
b1	29.9 (24.0-33.4)	24.9 (17.4-30.9)	37.1 (24.2-74.6)	<0.5

While particular embodiments of the invention have been described in detail, it will be apparent to those skilled in the art, that these embodiments are exemplary rather than limiting, and the true scope of the invention is that defined in the following claims.